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Crystallization and preliminary X-ray crystallographic studies of human 2′,3′-cyclic nucleotide 3′-phosphodiesterase

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The catalytic fragment of human 2′,3′-cyclic nucleotide 3′-phosphodiesterase (hCNP-CF) has been crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 300 as the precipitating agent. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 44.39, b = 55.35, c = 78.76 Å. There is one molecule per asymmetric unit. The crystals diffract to at least 1.8 Å resolution using synchrotron radiation and are suitable for X-ray structure analysis at high resolution.

1. Introduction

2′,3′-Cyclic nucleotide 3′-phosphodiesterase (CNP; EC 3.1.4.37) is firmly bound to membrane structures of brain white matter (Kurihara & Tsukada, 1967) and is mainly found in the central nervous system of vertebrates, especially of the amphibia and higher vertebrates (Kasama-Yoshida *et al.*, 1997). CNP catalyses the hydrolysis of 2′,3′-cyclic nucleotides to produce 2′-nucleotides *in vitro*, but the biological function of CNP *in vivo* remains unknown. Several functional studies imply possible functions of CNP. Isoprenylation of rat CNP demonstrated that the C-terminal domain of CNP is important for membrane binding and localization (Braun *et al.*, 1991). Overexpression of CNP in transgenic mice induced aberrant myelination (Gravel *et al.*, 1996). Recent studies on CNP-knockout mice revealed that disruption of the CNP gene causes hydrocephalus and premature death (Lappe-Siefke *et al.*, 2003). cDNA cloning demonstrated that human CNP isoform 1 (hCNP1) contains 401 amino-acid residues and has a MW of 45 kDa (Kurihara *et al.*, 1988). Kurihara *et al.* (1987) reported that bovine CNP isoform 1 is solubilized from membrane by cleavage at the carboxyl sides of residues 149 and 385 with elastase to yield the catalytic fragment of CNP (CNP-CF), which has intact CNP activity. Recently, CNP has been identified as a member of the 2H phosphoesterase superfamily (phosphoesterases with two conserved histidines; Mazumder *et al.*, 2002). The three-dimensional structures of plant CPDase (Hofmann *et al.*, 2000) and bacterial RNA ligase (Kato *et al.*, 2003) belonging to the 2H phosphoesterase superfamily have been reported. However, high-resolution crystal structures of mammalian enzymes belonging to the superfamily have not been reported. Thus, the three-dimensional structure of CNP is essential not only to understand the biological function of CNP but also to clarify the common catalytic mechanism of the enzymes belonging to the 2H phosphoesterase superfamily. Recently, Kozlov and coworkers reported the site-directed mutagenesis studies, ligand-binding-induced chemical shift change analysis and medium-resolution NMR model of the catalytic fragment of rat CNP (rCNP-CF, residues 164–378 of rCNP, which have 84% amino-acid sequence identity with residues 165–379 of hCNP1; Kozlov *et al.*, 2003). However, there are some discrepancies between the results of the functional studies of rCNP-CF and the NMR model of rCNP-CF. A prominent example is the interpretation of the ligand-binding-induced chemical shift change of Gly324 in rCNP-CF. NMR titrations showed large ligand-binding-induced chemical shift changes in the residues around Gly324. The authors interpreted these changes as being the result of conformational changes on ligand binding, because the NMR model showed that Gly324 was located far from the active site. Interestingly, mutation of the non-active-site residue Gly324 to alanine resulted in a fivefold increase in K_m and a 37-fold decrease in k_{cat} (Kozlov *et al.*, 2003). It is unlikely that the mutation of the non-active-site residue affected the catalytic efficiency of the enzyme this drastically. Thus, the high-resolution structure of CNP is essential to clarify the structure–function relationship of the enzyme. Here, we report the crystallization and preliminary X-ray diffraction analysis of the catalytic fragment of human CNP (hCNP-CF).

2. Methods and results

2.1. Expression and purification

hCNP-CF, consisting of residues 151–386 of hCNP1 (residues 171–406 of hCNP2) was cloned into a glutathione *S*-transferase (GST) gene-fusion vector pGEX-6P (Amersham) and

transformed into *Escherichia coli* strain BL21(DE3) (Novagen). Bacterial cultures were grown in M9 medium at 303 K to an OD_{600} of 0.6. Expression of hCNP-CF was induced by 0.5 m*M* IPTG for 20 h at 293 K. After this period, cells were harvested by centrifugation at 8000*g* for 15 min and the cell pellet was frozen at 253 K. Cell-lysis buffer [0.1 m*M* EDTA, 12.5 m*M* $MgCl_2$, 10%(*v*/*v*) glycerol, 100 m*M* KCl, 0.1% NP-40 and 1 m*M* DTT in 25 m*M* HEPES buffer pH 7.5] was added to the frozen cell pellets. Resuspended cells expressing fusion proteins were disrupted using ultrasonication on ice ten times, each for 1 min. The cell lysate was clarified by centrifugation at 15 000*g* and applied onto a 10 ml glutathione Sepharose 4B column (Amersham) equilibrated with cell-lysis buffer. The column was washed with 20 column volumes of cell-lysis buffer. After washing, the column was equilibrated with cleavage buffer (150 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT in 50 m*M* Tris buffer pH 7.0). The GST-fusion hCNP-CF bound to the column was digested with PreScission Protease (Amersham). After cleavage of the GST portion with the protease, fractions containing the GST-free hCNP-CF were applied onto another glutathione Sepharose 4B column to remove uncleaved GST-fusion hCNP-CF. Finally, the GST-free hCNP-CF was further purified by gel chromatography using a Superdex 75 pg column (Amersham) equilibrated with cleavage buffer. The fractions containing hCNP-CF were pooled and concentrated using a Centriplus-10 (Millipore) to 1 mg ml^{-1} and stored at 253 K after addition of glycerol to 20%(*v*/*v*). The specific activity of the recombinant hCNP-CF was 8.16×10^3 µmol min^{-1} per milligram of protein, whereas that of the recombinant full-length hCNP was 5.55×10^3 µmol min^{-1} per milligram of protein (Ichimiya *et al.*, unpublished data). As the molecular-weight ratio of hCNP-CF to full-length hCNP is 1:1.69, the specific activity of the hCNP-CF is considered to be comparable with that of the full-length hCNP.

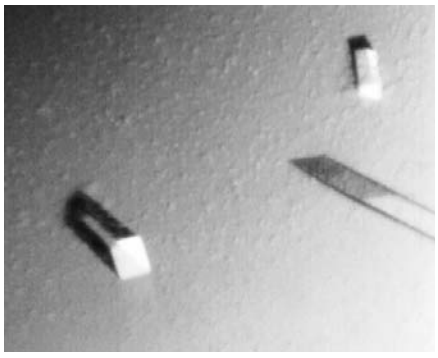

Figure 1
Orthorhombic crystals of hCNP-CF. Their approximate dimensions are 0.02 × 0.02 × 0.1 mm.

2.2. Crystallization

A stock solution of 0.8 mg ml^{-1} hCNP-CF with 120 m*M* NaCl, 0.8 m*M* EDTA, 0.8 m*M* DTT and 20%(*v*/*v*) glycerol in 40 m*M* Tris buffer pH 7.0 was dialyzed against 50 m*M* NaCl in 20 m*M* Tris buffer pH 7.5 and concentrated using a Centricon-10 (Millipore), yielding a working solution of 5 mg ml^{-1} hCNP-CF with 50 m*M* NaCl in 20 m*M* Tris buffer pH 7.5. Crystallization was carried out at 293 K by the hanging-drop vapour-diffusion method. The crystallization conditions found by the factorial approach (Jancarik & Kim, 1991) were optimized in order to obtain larger crystals. In the best case, a droplet was prepared by mixing equal volumes (1.5 + 1.5 µl) of the 5 mg ml^{-1} protein solution described above and reservoir solution consisting of 40%(*v*/*v*) polyethylene glycol with a mean molecular weight of 300 (PEG 300) in 100 m*M* phosphate–citrate buffer pH 4.2 and was equilibrated against 500 µl reservoir solution. Rod-shaped crystals with typical dimensions of about 0.02 × 0.02 × 0.1 mm were grown in two weeks (Fig. 1).

2.3. X-ray data collection

Since the crystallization conditions of hCNP described above contained 40%(*v*/*v*) PEG 300 in the reservoir solutions, X-ray data collections could be performed under cryogenic conditions without further addition of cryoprotectant. Data collection was performed by the rotation method at 100 K using an ADSC Q210 CCD detector with synchrotron radiation [λ = 1.00 Å at beamline NW12 of the PF-AR (Photon Factory Advanced Ring), Japan]. The Laue group and unit-cell parameters were determined using the *DPS* program package (Rossmann & van Beek, 1999). The Laue group was found to be *mmm* and the unit-cell parameters were $a = 44.39$, $b = 55.35$, $c = 78.76$ Å, $\alpha = \beta = \gamma = 90^\circ$. Only reflections with $h = 2n$, $k = 2n$ and $l = 2n$ were observed along the (h00), (0k0) and (00l) axes, respectively, indicating the orthorhombic space group $P2_12_12_1$. Assumption of one molecule (26.3 kDa) per asymmetric unit leads to an empirically acceptable V_M value of 1.84 Å^3 Da^{-1}, corresponding to a solvent content of 33% (Matthews, 1968). The current best diffraction data from a native crystal were collected to 1.8 Å resolution (R_{merge} and completeness of 6.2 and 98.2%, respectively) and processed with the program packages *DPS* (Rossmann & van Beek, 1999) and *CCP*4 (Collaborative Computational Project, Number 4, 1994) (Table 1). Although the size of the crystal used for the data collection was small (0.02 × 0.02 × 0.1 mm), data could be collected to 1.8 Å resolution. This is consistent with the fact that the solvent content of hCNP-CF crystals is relatively low and hCNP-CF molecules are assumed to be tightly packed in the unit cell. Initially, we tried to solve the structure of hCNP-CF by the molecular-replacement method. The crystal structures of plant CPDase (Hofmann *et al.*, 2000) and bacterial RNA ligase (Kato *et al.*, 2003), which have ∼10% amino-acid sequence identity to hCNP-CF, and the NMR model of rCNP-CF, which has ∼84% amino-acid sequence identity to hCNP-CF, were used as search models. Since these attempts failed, a search for heavy-atom derivatives intended for use in phasing by the multiple isomorphous replacement is under way.

Table 1
Crystallographic data of hCNP-CF.

Values in parentheses are for the outer resolution shell.

X-ray source	PF-AR NW12
Wavelength (Å)	1.00
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 44.39$, $b = 55.35$, $c = 78.76$
No. subunits per AU	1
Solvent content (%)	33
Resolution (Å)	1.8 (1.9–1.8)
No. unique reflections	18263
Multiplicity	6.5 (4.9)
Mean $\langle I/\sigma(I)\rangle$	9.4 (3.8)
R_{merge} (%)	6.2 (21.3)
Completeness (%)	98.2 (92.0)

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References

Braun, P. E., De Angelis, D., Shtybel, W. W. & Bernier, L. (1991). *J. Neurosci. Res.* **30**, 540–544.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Gravel, M., Peterson, J., Yong, V. W., Kottis, V., Trapp, B. & Braun, P. E. (1996). *Mol. Cell. Neurosci.* **6**, 453–466.
Hofmann, A., Zdanov, A., Genschik, P., Ruvinov, S., Filipowicz, W. & Wlodawer, A. (2000). *EMBO J.* **19**, 6207–6217.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kasama-Yoshida, H., Tohyama, Y., Kurihara, T., Sakuma, M., Kojima, H. & Tamai, Y. (1997). *J. Neurochem.* **69**, 1335–1342.

Kato, M., Shirouzu, M., Terada, T., Yamaguchi, H., Murayama, K., Sakai, H., Kuramitsu, S. & Yokoyama, S. (2003). *J. Mol. Biol.* **329**, 903–911.
Kozlov, G., Lee, J., Elias, D., Gravel, M., Gutierrez, P., Ekiel, I., Braun, P. E. & Gehring, K. (2003). *J. Biol. Chem.* **278**, 46021–46028.
Kurihara, T., Fowler, A. V. & Takahashi, Y. (1987). *J. Biol. Chem.* **262**, 3256–3261.
Kurihara, T., Takahashi, Y., Nishiyama, A. & Kumanishi, T. (1988). *Biochem. Biophys. Res. Commun.* **152**, 837–842.
Kurihara, T. & Tsukada, Y. (1967). *J. Neurochem.* **14**, 1167–1174.
Lappe-Siefke, C., Goebbels, S., Gravel, M., Nicksch, E., Lee, J., Braun, P. E., Griffiths, I. R. & Nave, K. A. (2003). *Nature Genet.* **33**, 366–374.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mazumder, R., Iyer, L. M., Vasudevan, S. & Aravind, L. (2002). *Nucleic Acids Res.* **30**, 5229–5243.
Rossmann, M. G. & van Beek, C. G. (1999). *Acta Cryst.* D**55**, 1631–1640.